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Screening and optimization of high-efficiency H_2-producing *Chlorella* strains

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ABSTRACT

Hydrogen (H_2) is a promising clean energy carrier that can be produced from green algae. By screening H_2-producing *Chlorella* species, *Chlorella* sp. ChiW1, isolated from a rice paddy field in Chai Nat province, Thailand, exhibited the highest H_2 production rate and yield. The mid-logarithmic phase cells of *Chlorella* sp. ChiW1 demonstrated the highest H_2 production. Nitrogen deprivation doubled the H_2 production compared to normal conditions, due to increased hydrogenase activity resulting from reduced O_2 evolution. Furthermore, a high concentration of acetic acid in Tris-acetate phosphate medium, combined with increased light intensity, significantly enhanced H_2 production. Under optimal conditions, nitrogen-deprived *Chlorella* sp. ChiW achieved a maximum H_2 production rate of 31.28 ± 1.73 µmol H_2 mg chl^{-1} h^{-1} and a total H_2 production yield of 925.32 ± 19.95 µmol H_2 mg chl^{-1} after 96 hours of light anaerobic incubation. Compared to other reported *Chlorella* strains, *Chlorella* sp. ChiW exhibited significantly higher H_2 production, underscoring its potential for efficient biohydrogen production.

1. INTRODUCTION

Currently, energy is crucial for advancing every aspect of life and driving progress in agriculture, industry, and daily activities. Global energy demand continues to rise each year, with fossil fuels serving as the primary energy source. However, fossil fuels are rapidly being depleted, and their consumption releases significant air pollutants, particularly greenhouse gases, which contribute to global climate change. Hydrogen (H_2) has gained significant attention as an alternative energy carrier. When consumed, H_2 has a heating value of 144 MJ kg^{-1} [1]. Additionally, the only byproduct of burning H_2 with oxygen (O_2) is water, making H_2 a clean and renewable energy carrier. H_2 can be produced through various processes, including steam-methane reforming, water electrolysis, and biological processes.

H_2 production through biological processes can occur in various microorganisms, including bacteria, cyanobacteria, and green algae. These microorganisms use various pathways facilitated by specific enzymes. H_2 production by green algae presents significant challenges that span biological, technological, and economical dimensions. H_2 production by green algae is influenced by the type of algal strain and the prevailing environmental conditions [2]. In Thailand, H_2 production by several green algal strains isolated from various water resources has been investigated [2–5]. However, green algal strains isolated from different geographic locations exhibit variations in robustness to environmental stresses, such as temperature fluctuations, nutrient variability, and high light intensity, which can hinder consistent H_2 production [6].

Nutrient deprivation is a factor influencing H_2 production in green algae. In *Chlorella prototheocoides*, under nitrogen and sulfur-deficient conditions, cells reduce photosystem II (PSII) activity, leading to decreased O_2 evolution. This reduction alleviates the inhibitory effects of O_2 on hydrogenase activity, thereby increasing H_2 production [7,8]. Additionally, potassium deprivation has been shown to enhance H_2 production in *Scenedesmus* sp. KMITL-OVG1 [9]. Furthermore, various external factors, such as medium pH, incubation temperature, and light intensity, play a critical role in optimizing H_2 production by green algae [3,4,10].

In this study, *Chlorella* strains were isolated from water sources in Thailand, selected, and evaluated for their H_2 production capabilities. The selected strain was optimized for H_2 production by studying various environmental parameters, including physical factors such as cell age, cell density, light intensity, temperature, and medium pH. Chemical factors, such as nutrient deprivation and the types and concentrations of carbon sources, were adjusted to assess their effects on H_2 production. These optimizations improved the H_2 production of the selected green algal strain, demonstrating the potential of the newly isolated algae for highly efficient biohydrogen production.

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2 . MATERIALS AND METHODS

2.1. *Chlorella* Strains and Cultivation

Twenty-two *Chlorella* strains were isolated from various water sources, including rice paddy fields, freshwater ponds, waterfalls, and natural seawater, across six provinces in the central regions and northeastern regions of Thailand: Bangkok, Chai Nat, Chanthaburi, Nakorn Nayok, Nakorn Ratchasima, and Nakorn Sawan. These *Chlorella* strains were previously identified based on morphological characteristics and genetic analysis using 18S rRNA sequencing. The algal cells were cultivated in a 250-ml flask containing 100 ml of Tris-acetate phosphate (TAP) medium (pH 7.2), which included 17.5 mM acetic acid as a carbon source [11]. The cultures were incubated at 30°C, shaken at 120 rpm, and exposed to a white, fluorescent light intensity of 30 µmol photons m^{-2} s^{-1} for 3 days.

2.2. Screening of High H_2-Producing *Chlorella* Strain

Chlorella strains, with an initial cell concentration at OD_{750} of approximately 0.1, were grown under the previously described conditions at 30°C for 36 hours. Cells were subsequently harvested by centrifugation at 8,000 × g, washed twice, and resuspended in fresh TAP medium. The cell density was adjusted to OD_{750} at approximately 2.0. Five ml of algal cell suspension was transferred into a glass vial and sealed with a rubber septum. To eliminate O_2 from the system, the algal cells in the vial were purged with argon gas for 15 minutes before being shaken at 120 rpm under a light intensity of 30 µmol photons m^{-2} s^{-1} at 30°C for 5 days. H_2 gas in the headspace was measured using a gas chromatograph (GC) every 24 hours of light incubation.

2.3. Measurements of OD_{750}, Total Chlorophyll Concentration, and Total Cell Number

The optical density of *Chlorella* was measured at a wavelength of 750 nm using a spectrophotometer (Shimadzu UV-1601, Japan). For total chlorophyll extraction, the cell culture was harvested by centrifugation at 8,000 × g at 4°C for 10 minutes, and the supernatant was discarded. One ml of 90 % (v/v) methanol was added to the cell pellet. The mixture was thoroughly mixed by vortexing and incubated at 70°C for 2 hours. Total chlorophyll content was measured using a spectrophotometer at wavelengths of 650 and 665 nm and calculated according to Becker [12]. The total cell number was determined by a hemocytometer (BOECO, Germany).

2.4. Effect of Cell Age and Cell Optical Density on H_2 Production

Chlorella was cultivated in TAP medium under previously described conditions at 30°C for 12, 24, 36, 48, and 60 hours. The cells were then harvested, washed, and resuspended in a fresh TAP medium. Five ml of algal cell suspension was transferred into a glass vial, and O_2 was eliminated from the system by purging with argon gas for 15 minutes. H_2 production was measured after 2 hours and then every 24 hours of anaerobic light incubation. To investigate the effect of cell optical density on H_2 production, the cell density of *Chlorella* was adjusted to OD_{750} at 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, and 3.0.

2.5. Effect of Nutrient Deprivation on H_2 Production

Chlorella was cultivated in TAP medium under previously described conditions at 30°C for 36 hours. The cells were harvested by centrifugation and resuspended in 100 ml of various modified media: normal TAP, potassium-deprived TAP (TAP-K), nitrogen-deprived TAP (TAP-N), phosphorus-deprived TAP (TAP-P), and sulfur-deprived TAP (TAP-S). TAP-K was prepared by removing KH_2PO_4 and K_2HPO_4 from TAP and adding phosphate in the form of NaH_2PO_4 and Na_2HPO_4, respectively, instead. TAP-N was prepared by removing NH_4Cl from TAP. TAP-P was prepared by removing KH_2PO_4 and K_2HPO_4 from TAP and adding potassium in the form of KCl. TAP-S was prepared by removing $MgSO_4.6H_2O$, $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$, and $CuSO_4.5H_2O$, and adding sulfate in the form of $MgCl_2$, $FeCl_2$, $ZnCl_2$, and $CuCl_2$, respectively. The cell suspensions were shaken on a rotary shaker at 120 rpm under light at 30°C for 24 hours. Cells were harvested by centrifugation, washed, and resuspended in fresh media of the same type. Five ml of algal cell suspension with an OD_{750} of 2.0 was transferred into a glass vial. H_2 production was measured under light anaerobic conditions using a GC.

2.6. Effect of Carbon Source and Concentration on H_2 Production

Chlorella was cultivated in TAP medium at 30°C for 36 hours before harvesting cells by centrifugation. The cells were then resuspended in nutrient-deprived media and incubated for 24 hours. Subsequently, cells were harvested again and resuspended in fresh nutrient-deprived TAP media containing different carbon sources: acetic acid, sodium acetate, glucose, sucrose, ethanol, propanol, butanol, and glycerol, all at the same C-atom molar concentration (35 mmol C-atom l^{-1}). The optical density of the cell suspension was adjusted to an OD_{750} of 2.0. Five ml of the cell suspension was transferred into a glass vial, sealed with a rubber septum, and purged with argon for 15 minutes before measuring H_2 production. To investigate the effect of carbon concentration on H_2 production, the carbon concentrations varied from 0 to 1,750 mmol C-atom l^{-1}.

2.7. Effect of Temperature, Initial Medium pH, and Light Intensity on H_2 Production

Chlorella adapted in nutrient-deprived media for 24 hours were harvested by centrifugation and resuspended in fresh nutrient-deprived media containing the selected carbon source and concentration before being transferred into vials. To study the effect of initial medium pH on H_2 production, the medium pH was adjusted to 5.0, 6.0, 7.0, 7.2, 8.0, and 9.0. To examine the effects of temperature and light intensity on H_2 production, the vials were incubated at temperatures of 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C and exposed to light intensities of 0, 30, 90, 150, 210, 300, and 390 µmol photon m^{-2} s^{-1}. H_2 production was measured every 24 hours using GC.

2.8. H_2 and O_2 Measurement

H_2 and O_2 levels in the headspace gas phase were measured using a GC (Hewlett-Packard HP5890A, Japan). A 0.5 ml sample of headspace gas was collected with a gas-tight syringe and injected into the GC. H_2 and O_2 were separated in a packed column with a molecular sieve (5 °A, 60/80 mesh), using argon as the carrier gas, and detected by a Thermal Conductivity Detector (TCD). The GC-TCD conditions were as previously reported [13]. H_2 and O_2 production was expressed as the amount of hydrogen produced (µmol H_2) per chlorophyll content (mg chl). The production rate was expressed as the amount of production per incubation time (hour).

2.9. *In Vivo* Hydrogenase Activity Measurement

In vivo H_2ase activity was assayed in a 12.5 ml glass-tight vial. The reaction mixture consisted of 25 mM potassium phosphate buffer containing 1% (v/v) Triton X-100, 5 mM methyl viologen, 20 mM

sodium dithionate, and the *Chlorella* culture [14]. The reaction mixture was incubated at 30°C in darkness for 30 minutes before H_2 production was measured using a GC. Hydrogenase activity was expressed as the amount of H_2 produced (μmol H_2) per chlorophyll content (mg chl) per incubation time in minutes.

2.10. Statistical Analysis

Data from each experiment were analyzed using one-way analysis of variance (ANOVA) with IBM SPSS Statistics version 28.0 (SPSS software, New York, NY). Results are presented as mean ± SE. The Duncan multiple range test was employed to identify statistically significant differences between the parameters under study, with the level of significance set at a p-value < 0.05. Different English letters in figures or tables indicate significant differences.

3. RESULTS

3.1. Screening of High H_2-Producing *Chlorella* Strain

In this study, all 22 strains of *Chlorella* exhibited H_2 production under anaerobic light conditions. Among them, *Chlorella* sp. ChiW1, isolated from a rice paddy field in Chai Nat province, showed the highest H_2 production rate with 5.07 ± 0.23 μmol H_2 mg chl^{-1} h^{-1} and reached a maximum H_2 production of 169.46 ± 7.48 μmol H_2 mg chl^{-1} after 48 hours of incubation in TAP medium (Table 1). This result indicates that each *Chlorella* strain exhibited different H_2 production rates. Notably, *Chlorella* sp. ChiW1 showed the highest H_2 production rate, while *Chlorella* sp. KLM146 exhibited the lowest rate, with an H_2 production rate 11.3 times lower. Therefore, *Chlorella* sp. ChiW1 was selected as the highest H_2-producing *Chlorella* strain for optimization of H_2 production.

3.2. Effect of Cell Age and Cell Density on H_2 Production by *Chlorella* sp. ChiW1

Chlorella sp. ChiW1, cultured for 36 hours in the mid-logarithmic phase, demonstrated the highest H_2 production rate with 5.12 ± 0.13 μmol H_2 mg chl^{-1} h^{-1} and a maximum H_2 production of 169.58 ± 9.77 μmol H_2 mg chl^{-1} after 48 hours of incubation in TAP medium under anaerobic light conditions (Fig. 1). Cells grown in the lag (12 hours) and early logarithmic (24 hours), late logarithmic (48 hours), and stationary (60 hours) phases showed lower H_2 production rates. This result indicates the significant role of cell age on H_2 production in *Chlorella* sp. ChiW1.

To investigate the effect of cell density on H_2 production, H_2 production was measured in cell cultures of *Chlorella* sp. ChiW1 was grown for 36 hours and adjusted to various OD_{750} values. Cultures with higher cell densities contained higher total cell numbers and total chlorophyll concentrations (Table 2). The *Chlorella* sp. ChiW1 culture with an OD_{750} of 2.0 exhibited the highest H_2 production rate with 5.07 ± 0.18 μmol H_2 mg chl^{-1} h^{-1} and a maximum H_2 production of 169.60 ± 6.80 μmol H_2 mg chl^{-1} at 48 hours of incubation (Table 2). H_2 production in *Chlorella* sp. ChiW1 increased with increasing cell densities, total cell numbers, and chlorophyll contents (Table 2). However, H_2 production decreased in cultures with OD_{750} values higher than 2.0. For further investigation on the optimization of H_2 production, *Chlorella* sp. ChiW1 with a cell age of 36 hours and an OD_{750} of 2.0 was chosen.

Table 1. H_2 production rate and maximum H_2 production yield of *Chlorella* sp. isolated from various water sources in Thailand.

Strain	Source	H_2 production rate (μmol H_2 mg chl^{-1} h^{-1})	Maximum H_2 production yield (μmol H_2 mg chl^{-1})
Chlorella sp. 2TK	Rice paddy field in Nakorn Sawan	1.42 ± 0.12^{i}	33.99 ± 2.81^{lm}
Chlorella sp. ChiS4	Rice paddy field in Chai Nat	0.61 ± 0.07^{k}	21.24 ± 5.61^{n}
Chlorella sp. ChiW1	Rice paddy field in Chai Nat	5.07 ± 0.23^{a}	169.46 ± 7.48^{a}
Chlorella sp. HNR141	Waterfall in Nakorn Nayok	1.93 ± 0.02^{h}	47.48 ± 0.35^{jk}
Chlorella sp. HNR143	Waterfall in Nakorn Nayok	4.19 ± 0.11^{c}	100.65 ± 2.53^{de}
Chlorella sp. HNR146	Waterfall in Nakorn Nayok	2.38 ± 0.16^{g}	86.00 ± 10.48^{fg}
Chlorella sp. HNR147	Waterfall in Nakorn Nayok	2.91 ± 0.44^{ef}	69.86 ± 10.55^{hi}
Chlorella sp. KLM143	Waterfall in Nakorn Ratchasima	3.49 ± 0.07^{d}	157.76 ± 3.17^{b}
Chlorella sp. KLM144	Waterfall in Nakorn Ratchasima	4.54 ± 0.29^{b}	108.99 ± 47.06^{d}
Chlorella sp. KLM145	Waterfall in Nakorn Ratchasima	1.80 ± 0.17^{h}	43.12 ± 4.06^{jkl}
Chlorella sp. KLM146	Waterfall in Nakorn Ratchasima	0.45 ± 0.05^{k}	31.51 ± 2.79^{m}
Chlorella sp. KMITL CirG	Freshwater in Bangkok	1.98 ± 0.28^{h}	46.27 ± 6.78^{jk}
Chlorella sp. LSD-W1	Seawater in Chanthaburi	1.23 ± 0.14^{ij}	29.60 ± 3.28^{mn}
Chlorella sp. RSS141	Freshwater in Nakorn Ratchasima	1.14 ± 0.13^{ij}	38.73 ± 0.70^{klm}
Chlorella sp. RSS147	Freshwater in Nakorn Ratchasima	3.47 ± 0.09^{d}	83.32 ± 2.17^{fg}
Chlorella sp. SRK141	Waterfall in Nakorn Nayok	3.01 ± 0.27^{ef}	120.82 ± 5.96^{c}
Chlorella sp. SRK149	Waterfall in Nakorn Nayok	2.73 ± 0.25^{f}	65.71 ± 6.00^{i}
Chlorella sp. SWT141	Waterfall in Nakorn Ratchasima	1.75 ± 0.04^{h}	76.00 ± 6.40^{gh}
Chlorella sp. SWT142	Waterfall in Nakorn Ratchasima	2.30 ± 0.05^{g}	157.22 ± 11.58^{b}
Chlorella sp. SWT144	Waterfall in Nakorn Ratchasima	3.06 ± 0.06^{e}	156.93 ± 4.96^{b}
Chlorella sp. SWT146	Waterfall in Nakorn Ratchasima	2.30 ± 0.04^{g}	93.48 ± 3.17^{ef}
Chlorella sp. WTK	Waterfall in Nakorn Nayok	1.09 ± 0.10^{j}	52.32 ± 4.8^{j}

Different letters indicate statistically significant differences ($p < 0.05$) using one-way ANOVA.

3.3. Effect of Nutrient Deprivation on H_2 Production by *Chlorella* sp. ChiW1

To enhance H_2 production, *Chlorella* sp. ChiW1 cells grown in TAP medium for 36 hours were adapted in different nutrient-deprived TAP media for 24 hours before adjusting the cell density to an OD_{750} of 2.0. The highest H_2 production rate of 10.57 ± 0.39 µmol H_2 mg chl^{-1} h^{-1} was observed under nitrogen-deprived conditions (Table 3). *Chlorella* sp. ChiW1 incubated in TAP-N reached a maximum H_2 production of 321.18 ± 6.92 µmol H_2 mg chl^{-1} after 48 hours of incubation (Table 3). This H_2 production was approximately twice that observed under normal TAP conditions. Sulfur deprivation in TAP also promoted H_2 production in *Chlorella* sp. ChiW1, resulting in an H_2 production rate of 8.45 ± 049 µmol H_2 mg chl^{-1} h^{-1} and a maximum H_2 production of 270.36 ± 9.36 µmol H_2 mg chl^{-1}, about 1.6 times higher than under normal TAP conditions (Table 3). However, H_2 production in *Chlorella* sp. ChiW1 incubated in phosphorus-deprived and potassium-deprived media was not significantly different from that observed under normal TAP conditions (Table 3).

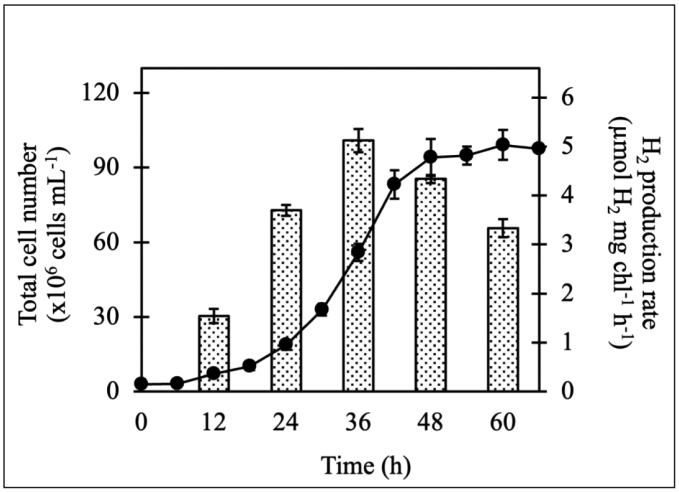

Figure 1. Growth by cell number measurement and H_2 production rate of *Chlorella* sp. ChiW1 cultivated in TAP medium for different cultivation times.

3.4. Effect of Nitrogen Deprivation on H_2 and O_2 Production, and *In Vivo* Hydrogenase Activity by *Chlorella* sp. ChiW1

To investigate the effects of nitrogen-deprived metabolism on H_2 production, *Chlorella* sp. ChiW1 cells were incubated in TAP-N medium under light aerobic conditions for 24 hours before harvesting cells to determine H_2 and O_2 production, as well as *in vivo* H_2ase activity under anaerobic conditions. The nitrogen-deprived cells produced approximately two-fold higher H_2 production and H_2ase activity than normal cells during 120 hours of light anaerobic conditions (Fig. 2A and B). In contrast, O_2 production was significantly decreased in nitrogen-deprived cells (Fig. 2C). The increased H_2 production was attributed to an increase in H_2ase activity, which resulted from the decreased O_2 in the system.

3.5. Effect of Carbon Source and Concentration on H_2 Production by N-Deprived Cells of *Chlorella* sp. ChiW1

H_2 production was measured in N-deprived cells of *Chlorella* sp. ChiW1 incubated in TAP-N medium containing various carbon

Table 2. H_2 production rate and maximum H_2 production yield of *Chlorella* sp. ChiW1 cultivated in TAP for 36 hours and adjusted the initial optical cell densities at 750 nm from 0.2 to 3.0. Each different initial OD_{750} showed different total cell numbers and total chlorophyll concentrations.

OD_{750}	Total cell number (×10 cells ml^{-1})	Total chlorophyll concentration (mg l^{-1})	H_2 production rate (µmol H_2 mg chl^{-1} h^{-1})	Maximum H_2 production yield (µmol H_2 mg chl^{-1})
0.2	3.11 ± 0.45^{i}	2.85 ± 0.20^{h}	1.75 ± 0.37^{e}	57.02 ± 7.45^{f}
0.4	6.21 ± 0.74^{h}	5.83 ± 0.72^{g}	2.26 ± 0.24^{d}	71.74 ± 4.34^{e}
0.6	9.29 ± 0.65^{g}	8.61 ± 0.54^{f}	3.06 ± 0.29^{c}	116.02 ± 3.32^{c}
0.8	11.96 ± 0.99^{f}	10.35 ± 0.96^{f}	3.23 ± 0.03^{c}	127.35 ± 7.52^{c}
1.0	14.57 ± 0.95^{e}	13.99 ± 0.22^{e}	4.32 ± 0.42^{b}	142.73 ± 10.99^{b}
1.5	20.14 ± 0.86^{d}	19.28 ± 0.63^{d}	4.37 ± 0.43^{b}	152.39 ± 9.00^{b}
2.0	27.96 ± 1.10^{c}	24.96 ± 1.54^{c}	5.07 ± 0.18^{a}	169.60 ± 6.80^{a}
2.5	34.14 ± 1.26^{b}	30.83 ± 0.93^{b}	3.99 ± 0.25^{b}	126.10 ± 6.06^{c}
3.0	42.68 ± 1.24^{a}	36.84 ± 2.70^{a}	3.12 ± 0.12^{c}	96.25 ± 8.36^{d}

Different letters indicate statistically significant differences ($p < 0.05$) among all data in the same row using one-way ANOVA.

Table 3. H_2 production rate and maximum H_2 production yield of 36 hours old *Chlorella* sp. ChiW1 cells incubated in different nutrient-deprived TAP media for 24 hours before adjusting the cell density to an OD_{750} of 2.0.

Type of medium	H_2 production rate (µmol H_2 mg chl^{-1} h^{-1})	Maximum H_2 production yield (µmol H_2 mg chl^{-1})
TAP	5.02 ± 0.25^{c}	166.50 ± 6.69^{c}
TAP-K	5.63 ± 0.18^{c}	179.61 ± 8.14^{c}
TAP-N	10.57 ± 0.39^{a}	321.18 ± 6.92^{a}
TAP-P	5.60 ± 0.29^{c}	182.81 ± 8.19^{c}
TAP-S	8.45 ± 049^{b}	270.36 ± 9.36^{b}

Different letters indicate statistically significant differences ($p < 0.05$) among all data in the same row using one-way ANOVA.

sources, including acetic acid, butanol, ethanol, glucose, glycerol, propanol, sodium acetate, and sucrose, all at the same C-atom concentration of 35 mmol C-atom l^{-1}. The result showed that *Chlorella* sp. ChiW1 incubated in TAP-N medium containing acetic acid as the carbon source exhibited the highest H_2 production rate with 10.90 ± 0.95 µmol H_2 mg chl^{-1} h^{-1} (Table 4) and reached a maximum H_2 production yield of 330.52 ± 15.80 µmol H_2 mg chl^{-1} after 72 hours of incubation (Table 4). On the other hand, the lowest H_2 production rates were observed in cells incubated in TAP-N medium containing either glycerol or sucrose (Table 4). Thus, acetic acid was identified as the most effective carbon source for H_2 production by *Chlorella* sp. ChiW1.

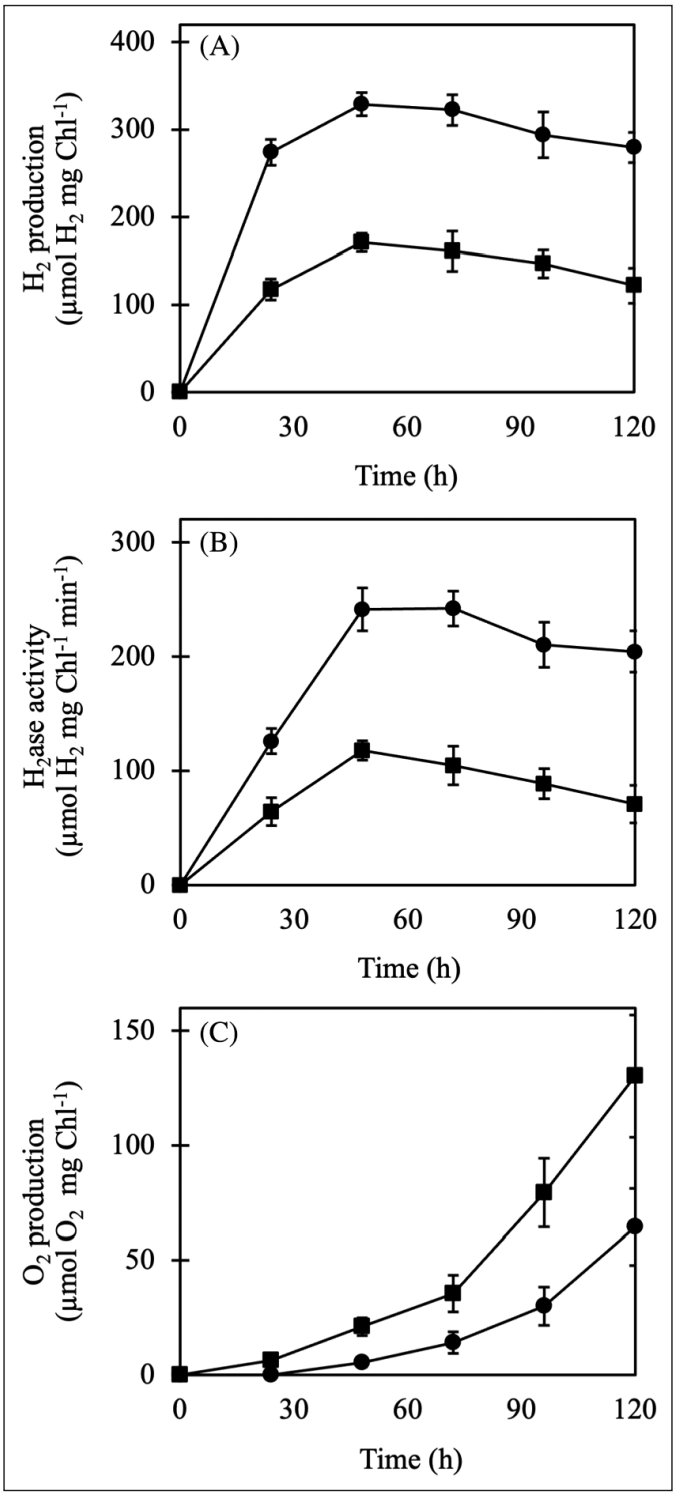

Figure 2. H_2 production (A), H_2ase activity (B), O_2 production (C) of *Chlorella* sp. ChiW1 cells incubated in normal TAP (■) and TAP-N medium (●).

Additionally, H_2 production was determined in nitrogen-deprived cells incubated in TAP-N medium containing various concentrations of acetic acid, with TAP-N containing 35 mmol C-atom l^{-1} acetic acid used as the control medium. The result showed that the maximum H_2 production rate of 14.66 ± 0.53 µmol H_2 mg Chl^{-1} h^{-1}, and maximum H_2 production yield of 445.31 ± 21.53 µmol H_2 mg Chl^{-1} were obtained in cells incubated in TAP-N containing 175 mmol C-atom l^{-1} acetic acid, which is five times the carbon concentration of TAP-N (Table 5). Concentrations lower or higher than this decreased H_2 production (Table 5). Without the addition of acetic acid, *Chlorella* sp. ChiW1 had the lowest H_2 production rate with only 0.34 ± 0.06 µmol H_2 mg Chl^{-1} h^{-1} (Table 5), indicating the significance of carbon source on H_2 metabolism in *Chlorella* sp. ChiW1.

3.6. Effect of Medium pH, Incubation Temperature, and Light Intensity on H_2 Production by *Chlorella* sp. ChiW1

H_2 production was measured in nitrogen-deprived *Chlorella* sp. ChiW1 cells were incubated in TAP-N medium containing 175 mmol C-atom l^{-1} acetic acid, with pH varied in the range of 5.0–9.0. The highest H_2

Table 4. H_2 production rate and maximum H_2 production yield of N-deprived adapted cells of *Chlorella* sp. ChiW1 incubated in TAP-N medium containing various carbon sources.

Carbon source	H_2 production rate (µmol H_2 mg chl^{-1} h^{-1})	Maximum H_2 production (µmol H_2 mg chl^{-1})
Acetic acid	10.90 ± 0.95[a]	330.52 ± 15.802[a]
Butanol	7.39 ± 0.67[c]	231.87 ± 23.24[c]
Ethanol	7.11 ± 0.42[c]	256.79 ± 16.611[c]
Glucose	4.64 ± 0.79[d]	174.57 ± 18.044[d]
Glycerol	2.27 ± 0.70[e]	81.41 ± 14.501[e]
Propanol	5.57± 0.65[d]	194.39 ± 17.049[d]
Sodium acetate	9.30 ± 0.70[b]	290.22 ± 21.893[b]
Sucrose	2.41 ± 0.33[e]	75.45 ± 10.136[e]

Different letters indicate statistically significant differences ($p < 0.05$) using one-way ANOVA.

Table 5. H_2 production rate and maximum H_2 production yield of N-deprived adapted cells of *Chlorella* sp. ChiW1 incubated in TAP-N medium containing acetic acid concentrations.

Concentration of acetic acid	H_2 production rate (µmol H_2 mg chl^{-1} h^{-1})	Maximum H_2 production (µmol H_2 mg chl^{-1})
0	0.34 ± 0.06[e]	80.69 ± 3.72[e]
17.5	7.81 ± 0.38[d]	224.65 ± 25.23[d]
35	10.21 ± 0.33[c]	341.84 ± 17.05[c]
70	12.02 ± 0.51[a]	389.99 ± 23.83[b]
175	14.66 ± 0.53[b]	445.31 ± 21.53[a]
350	13.00 ± 0.85[b]	320.54 ± 37.52[c]
1,750	8.84 ± 1.19[d]	212.18 ± 30.85[d]

Different letters indicate statistically significant differences ($p < 0.05$) using one-way ANOVA.

production rate with 14.46 ± 1.19 µmol H_2 mg Chl^{-1} h^{-1} was found in cells incubated at pH 7.2 (Fig. 3A). This rate did not show significant differences compared to the H_2 production rates of cells incubated at pH 7.0. Cells at pH 7.2 reached a maximum H_2 production yield of 454.94 ± 14.28 µmol H_2 mg Chl^{-1} after 96 hours of incubation. Under various incubation temperatures, *Chlorella* sp. ChiW1 cells incubated in TAP-N medium containing 175 mmol C-atom l^{-1} acetic acid with pH 7.2 at incubation temperature 35°C demonstrated the highest production rate with 16.89 ± 0.80 µmol H_2 mg Chl^{-1} h^{-1} (Fig. 3B) and reached the highest production rate with 517.42 ± 20.50 µmol H_2 mg Chl^{-1} after 96 hours of incubation. To investigate the effect of light intensity on H_2 production by *Chlorella* sp. ChiW1, light intensity was varied in the range of 0–390 µmol photons m^{-2} s^{-1}. The result indicated that cells incubated in TAP-N medium containing 175 mmol C-atom l^{-1} acetic acid with pH 7.2 at incubation temperature 35°C under a light intensity of 210 µmol photons m^{-2} s^{-1} showed the highest H_2 production rate with 31.28 ± 1.73 µmol H_2 mg Chl^{-1} h^{-1} (Fig. 3C) and achieved the maximum H_2 production yield of 952.38 ± 25.55 µmol H_2 mg Chl^{-1}. This H_2 production rate was approximately 2 times higher than that of cells incubated at 30 µmol photons m^{-2} s^{-1}. In contrast, cells incubated in darkness provided the lowest H_2 production rate with 3.20 ± 0.54 µmol H_2 mg Chl^{-1} h^{-1} (Fig. 3C).

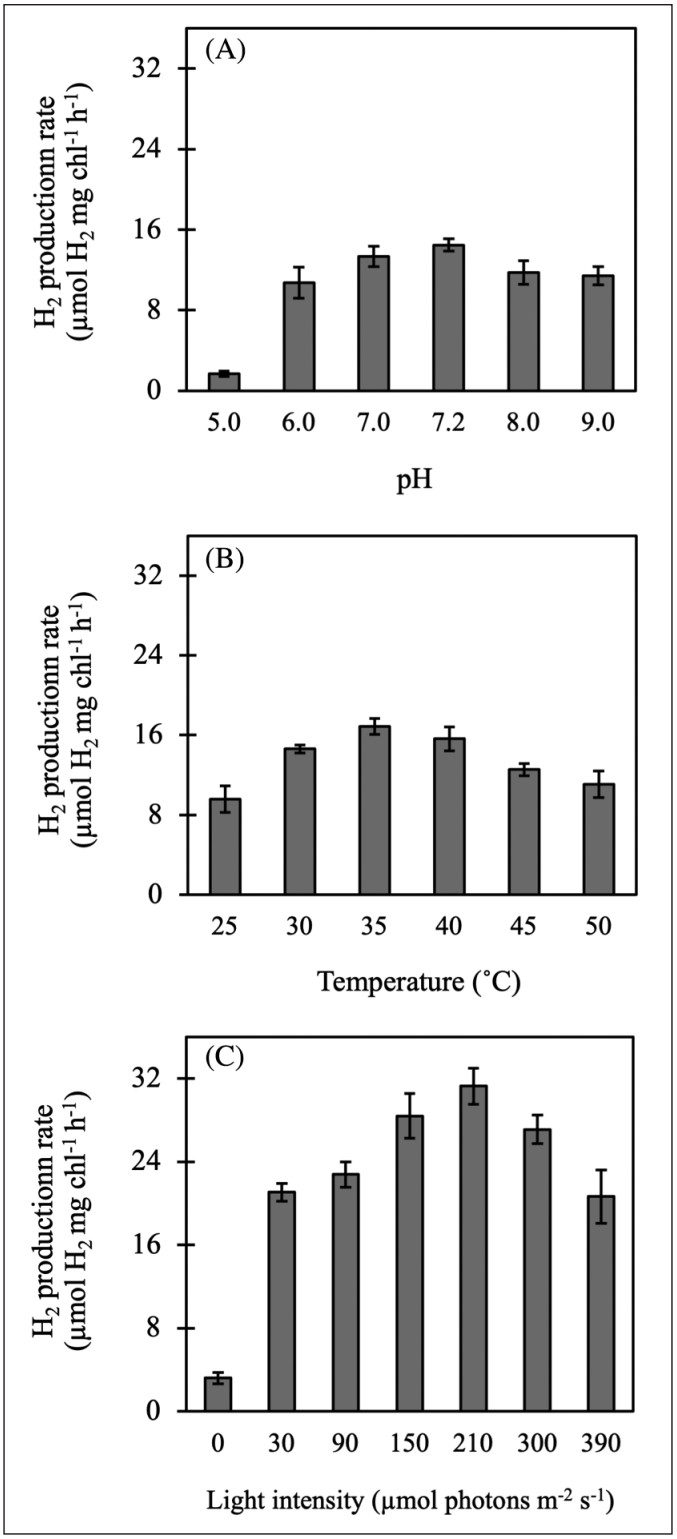

Figure 3. Effect of medium pH (A), incubation temperature (B), and light intensity (C) on H_2 production by *Chlorella* sp. ChiW1 incubated in TAP-N containing 175 mmol C-atom l^{-1} acetic acid.

4. DISCUSSION

4.1. Screening of Potential H_2-Producing *Chlorella* Strain

Among the 22 *Chlorella* strains investigated, *Chlorella* sp. ChiW1, isolated from a rice paddy field in Chai Nat province, exhibited the highest H_2 production rate and yield (Table 1). This could be attributed to the unique composition of water in rice paddy fields, which may contain primary macronutrients (N, P, and K) essential for both plant and algal growth [15]. Additionally, the presence of Mg ions, vital for photosynthesis [16], and Fe ions, which act as cofactors for the hydrogenase enzyme [17], likely contribute to this strain's efficiency. Moreover, rice paddy fields are exposed to high light intensities and elevated temperatures, which may have favored the development of *Chlorella* sp. ChiW1 with an enhanced photosynthetic rate. Its hydrogenase enzyme might also exhibit greater tolerance to O_2 compared to strains isolated from other water sources.

In Thailand, previous research focused on the isolation of green algae from diverse freshwater sources, resulting in the identification of 43 strains across six genera [2]. These strains exhibited variations in their capacity for H_2 production. Moreover, within the same genus, differences in H_2 production were observed under various TAP conditions. Most *Chlorella* strains and all *Chlamydomonas* strains enhanced H_2 production under simultaneous nitrogen limitation and sulfur deprivation conditions [2]. Additionally, a previous report on the isolation of green algae from rice paddies in Thailand, cultured in BG11 medium, identified nine green algal strains that demonstrated varying levels of H_2 production [15]. From screening H_2-producing green algae isolated from natural seawater in Thailand, *Chlorella* sp. LSD-W2 showed the highest H_2 production rate with 1.52 µmol H_2 mg chl^{-1} h^{-1} when incubated in TAP medium under light anaerobic conditions. Additionally, *Chlorella* sp. LSD-W2 under N-deprived conditions increased its H_2 production rate by up to 20 times compared to that under normal TAP conditions [5].

These findings underscore that variations in H_2 production can occur even within the same strain of green algae under similar or different cultivation conditions. This variability highlights the importance of not only the inherent characteristics of the algae strain but also the specific environmental and nutritional factors during cultivation. Despite these variations, our study specifically demonstrated that *Chlorella* sp. ChiW1 shows promising potential for H_2 production. Therefore, *Chlorella* sp. ChiW1 emerges as an intriguing candidate deserving further investigation and optimization of cultivation conditions and

other influencing factors to enhance H_2 production. By studying and optimizing these conditions and factors, such as cell age, cell density, nutrient availability, medium pH, incubation temperature, and light intensity, it may be possible to maximize the H_2 production capabilities of *Chlorella* sp. ChiW1, thus contributing to its potential application in sustainable biofuel production or other industrial processes.

4.2. Effect of Cell Age and Cell Density on H_2 Production by *Chlorella* sp. ChiW1

The suitability of algal cells is one of the first aspects studied for optimizing H_2 productivity. The age and density of algal cells were varied to determine optimal conditions for H_2 production by *Chlorella* sp. ChiW1. Experimental results indicated that *Chlorella* sp. ChiW1 produced H_2 most efficiently at a cell age of 36 hours (Fig. 1) and a cell density of OD_{750} equal to 2.0 (Table 2). At 36 hours of cultivation, *Chlorella* sp. ChiW1 was in the mid-logarithmic growth phase (Fig. 1), a stage where the cells grew well, divided very quickly, and produced high chlorophyll content. The chlorophyll content is a crucial factor for H_2 production by green algal cells under light conditions since chlorophyll is responsible for absorbing light energy used in the photosynthesis process at PSII. When chlorophyll absorbs light energy, it initiates the water-splitting reaction, producing protons, electrons, and oxygen (O_2). The electrons and protons generated from water splitting can be utilized for H_2 production, mediated by hydrogenase (H_2ase) [18]. Therefore, as chlorophyll content increased, the substrates for H_2 production also increased, leading to higher H_2 output. In *Chlamydomonas reinhardtii* UTEX 90, cells at the late-exponential phase containing a chlorophyll content of 39.29 mg l^{-1} showed the highest H_2 production of 159 ml H_2 g^{-1} cell under anaerobic sulfur-deprived conditions [19].

However, the water-splitting reaction in photosynthesis produces O_2 as a byproduct, which is a potent inhibitor of H_2ase [20]. Excessive chlorophyll content resulted in higher residual O_2 in the system, inhibiting enzyme activity. Thus, optimal cell concentration was critical to avoid excessive chlorophyll levels. This aligns with the experimental results showing that cell density higher than an OD_{750} of 2.0 resulted in decreased H_2 production despite the increased cell density. In addition, an excess number of cells might lead to lower electron availability from the photosynthetic process due to cell shading [18]. Previous research on the effect of cell density on H_2 production by *Scenedesmus* sp. KMITL-OVG1 demonstrated that H_2 production decreased from 0.79 to 0.48 ml l^{-1} h^{-1} when OD_{750} increased from 0.8 to 1.0 [9].

4.3. Effect of Nutrient Deprivation on H_2 Production

H_2 production by *Chlorella* sp. ChiW1 increased under N-deprived and S-deprived conditions (Fig. 2), consistent with several studies promoting H_2 production by green algae. Under nitrogen deficiency, algal cells adapt by altering their metabolism, for example, by inhibiting growth and protein synthesis while promoting starch accumulation [21–23]. When cells enter anaerobic conditions, starch is degraded into glucose molecules, which then generate numerous electrons that serve as substrates for H_2ase activity [24]. Consequently, H_2 production by algal cells under nitrogen deprivation is induced. Moreover, nitrogen deficiency also restricts the synthesis of the D1 protein, thereby inhibiting the repair of PSII [25]. This reduces PSII activity, leading to the establishment of anaerobiosis and the induction of H_2ase activity, eventually enhancing H_2 production [7,8]. Previous reports showed similar results with N-deprivation enhancing H_2 production by *Chlorella* sp. LSD-W2 and *Chlorella pyrenoidosa* IOAC707S [5,26]. Thus, N-deprived conditions, which generated the highest H_2 production by *Chlorella* sp. ChiW1, were selected for further optimization.

In addition to nitrogen deprivation, sulfur deficiency could also induce H_2 evolution in several green algal strains, including *Chlorella* sp. ChiW1. Sulfur, as a crucial component of amino acids, proteins, and enzymes, plays a significant role in cellular metabolism [27]. In *C. reinhardtii,* sulfur deprivation led to the rapid degradation of the PSII complex, which severely inhibited electron transport in PSII [28]. This reduction in PSII activity decreased O_2 photoevolution, thereby increasing H_2ase activity, and ultimately enhancing H_2 production.

Consistent with previous reports on *C. reinhardtii* CC-125 and *Chlorella* sp. LSD-W2, sulfur deprivation enhanced H_2 production compared to normal TAP conditions [5,29]. Incubating algal cells under nutrient-deprived conditions can reduce medium costs; however, it poses significant limitations when scaling up to large-scale systems. Scaling up nutrient deprivation processes requires precise control and additional resources to monitor and adjust nutrient levels, which increases operational costs and reduces the economic feasibility of large-scale H_2 production systems. Furthermore, prolonged nutrient deprivation can stress the cells, leading to reduced viability and potentially causing system instability. This is especially critical in open or semi-continuous systems where maintaining a stable culture is challenging.

4.4. H_2 Metabolism Under Nitrogen Deprivation

Under nitrogen deficiency, *Chlorella* sp. ChiW1 increased H_2 production and H_2ase activity but decreased O_2 production (Fig. 2). The decreased O_2 in the system might be caused by a lower O_2 evolution rate due to the reduced photosynthesis and increased respiration rate. Previous studies showed that *C. pyrenoidosa* and *C. protothecoides* exhibited increased respiration rates while O_2 evolution and the efficiency of PSII activity decreased under N-deprivation [7,8,30]. As a result, N-deprivation could prolong the anaerobic state of the incubation system and promote H_2ase activity.

Besides PSII, starch is the main endogenous electron source for H_2 production by green algae [8]. During nitrogen deprivation, intracellular starch in *Chlorella* sp. ChiW1 accumulates approximately 2–3 times higher than under normal conditions (data not shown). Under anaerobic conditions, cells degrade starch rather than accumulate it, subsequently releasing a large number of electrons. A previous study indicated that starch concentration increased in *C. reinhardtii* cells incubated in both sulfur-deprived and nitrogen-deprived media. Specifically, cells under nitrogen-deprived conditions demonstrated approximately twice the starch accumulation compared to those under sulfur-deprived conditions [21]. This increased starch accumulation was subsequently catabolized, releasing a high level of electrons, which could promote hydrogenase activity and enhance H_2 production.

4.5. Effect of Carbon Source and Concentration on H_2 Production by *Chlorella* sp. ChiW1

The carbon source and its concentration have been shown to play a crucial role in enhancing H_2 production by green algae. In *Chlorella* sp. ChiW1, acetic acid available in TAP medium was identified as a potential carbon source for H_2 production (Table 4), with the optimal acetic acid concentration at 175 mmol C-atom l^{-1} or 87.5 mM (Table 5). Acetic acid provided the highest H_2 production by *Chlorella* sp. ChiW1, suggesting that it might be easily metabolized in carbohydrate metabolism, ultimately establishing anoxia in the cultures through its

oxidation in the tricarboxylic acid cycle and oxidative phosphorylation. In *C. reinhardtii* strain 704 (cw15 arg7+ Nia1:Ars mt+), the addition of acetate to cultures decreased photosynthetic efficiency and promoted mitochondrial respiration, leading to a reduction in O_2 evolution [31].

Furthermore, studies on the fermentative metabolism in *C. reinhardtii* F-60 have supported the conversion of acetate into H_2 under anaerobic and light-dependent conditions via the citric acid and glyoxylate cycles [32]. Thus, the availability of acetic acid is assumed to reduce O_2 in the system and subsequently promote H_2ase activity. Several previous studies have reported that acetate as a carbon source provided the highest H_2 yield in *C. reinhardtii* strain CC124 and *Parachlorella kessleri* [33,34].

In addition, *Chlorella* sp. ChiW1 incubated in acetic acid-free TAP medium produced less H_2 than cells in an acetic acid-containing TAP medium (Table 5). This observation is consistent with previous studies that highlighted the importance of using acetate as a carbon source in culture media, compared to autotrophic cultivation. It was found that H_2 production by *C. reinhardtii* cultured in acetate-containing media was higher than that of autotrophic cultures [31]. The concentration of acetic acid is also crucial for H_2 production, as excessively high concentrations could affect various factors, such as pH balance and inhibition of initial substrates [35,36].

4.6. Effect of Medium pH, Incubation Temperature, and Light Intensity on H_2 Production by *Chlorella* sp. ChiW1

The pH of the culture medium significantly influences H_2 production by green algae, including various strains of *Chlorella*. In this study, the pH levels of 7.0 and 7.2 provided the highest H_2 production by *Chlorella* sp. ChiW1 (Fig. 3). This indicates that a neutral pH is optimal for H_2ase activity, whereas acidic and alkaline pH levels are not suitable. This finding is consistent with a study on *Chlorella* sp. KLSc59, which demonstrated that the highest H_2 production was found at pH 7.2 [4]. Other studies have reported that the maximum H_2 production by *Chlorella vulgaris* and other *Chlorella* species was obtained at pH 8.0 [37,38]. This suggests that most green microalgal strains including *Chlorella* sp. ChiW1, can efficiently produce H_2 within a range of neutral pH conditions, while inhibition of H_2-producing enzymes occurs in both acidic and extremely alkaline conditions [39]. In addition, maintaining a pH at the optimal level is another crucial factor for sustainable H_2 production in green algae [40].

Microorganisms can produce H_2 efficiently at different optimal temperatures, depending on the strain. The high H_2 production by *Chlorella* sp. ChiW1 occurred within the temperature range of 30°C–40°C, with the maximum H_2 production at 35°C (Fig. 3), suggesting this is the optimal temperature for H_2ase activity. However, *Chlorococcum minutum* and *C. reinhardtii* exhibited efficient H_2 production at 25°C, and a significant decrease in growth and H_2 production was obtained at 35°C [41]. In *Chlorella* sp. NIER-10003, higher temperatures reduced the adaptation period and increased the H_2 production rate. However, temperatures above 40°C were unsuitable, as the cells began to die. Conversely, temperatures below 25°C resulted in a prolonged adaptation period and a significantly reduced hydrogen production rate [47].

Light intensity plays a crucial role in H_2 production by green algae since electrons, which are substrates for H_2ase, are obtained from light energy via water photolysis. Theoretically, green algae can utilize sunlight as an energy source to generate H_2, reaching up to 13% efficiency [39,48]. H_2 production by *Chlorella* sp. ChiW1 increased with higher light intensities until it reached the optimal light intensity at 210 µmol photons m^{-2} s^{-1} (Fig. 3). Similarly, *C. reinhardtii* UTEX 90 cultivated in sulfur-deficient conditions demonstrated the impact of light on H_2 production, achieving the highest H_2 production under a light intensity of 200 µmol photons m^{-2} s^{-1} [49]. Increased light intensity affects internal mechanisms, one of which involves the inhibition of the synthesis of the D1 protein in PSII [50], contributing to the prolonged maintenance of an anaerobic system for higher H_2 production [51]. In

Table 6. H_2 production of various *Chlorella* strains under different incubation conditions.

Strains	Conditions	H_2 production rate	Maximum H_2 production	References
Chlorella sp. ChiW1	N-deprived TAP plus 175 mmol C-atom atom l^{-1} acetic acid, pH 7.2, 35˚C, 150 µmol photons m^{-2} s^{-1}	31.28 ± 1.73 µmol H_2 mg chl^{-1} h^{-1} or 15.32 ± 0.45 ml l^{-1} h^{-1}	925.32 ± 19.95 µmol H_2 mg chl^{-1} or 448.98 ± 5.77 ml l^{-1}	This study
Chlorella sp. KLSc59	TAP plus 1 mM ethanol, pH 7.2, 36˚C, 53.2 µmol photons m^{-2} s^{-1}	N/A	850 µmolH_2 mgchl^{-1}	[4]
Chlorella pyrenoidosa 707S	S-deprived modified marine plus 0.7 mM NH_4Cl, pH 7.8, 25±1˚C, 35–45 mE m^{-2} s^{-1} (14 hours light: 10 hours dark)	N/A	98.82 ± 16.95 ml l^{-1}	[42]
Chlorella sorokiniana Ce	S-deprived TAP, pH 7.2, 30˚C, 120 µmol photons m^{-2} s^{-1}	1.35 ml l^{-1} h^{-1}	N/A	[43]
Chlorella sorokiniana KU204	S-deprived TAP plus 0.7 mM NH4Cl, pH 7.3, 25˚C, 35 µmol photons	N/A	89.64 ml l^{-1}	[2]
Chlorella lewinii KU201	S-deprived TAP plus 0.7 mM NH4Cl, pH 7.3, 25˚C, 35 µmol photons m^{-2} s^{-1}	N/A	13.03 ml l^{-1}	[2]
Chlorella protothecoides Krueg	S-deprived TAP, 26˚C, 35–50 µmol photons m^{-2} s^{-1}	2.93 ml l^{-1} h^{-1}	123.6 ml l^{-1}	[44]
Chlorella protothecoides	N-, S-deprived TAP pH 7.3, 25˚C, 30–35 µmol photons m^{-2} s^{-1}	N/A	82.5 ml l^{-1}	[45]
Chlorella sp. LSD-W2	N-deprived TAP, 30¡C, 30 µmol photons m^{-2} s^{-1}	1.52 ± 0.09 µmol H_2 mg chl^{-1} h^{-1}	N/A	[5]
Chlorella sp. KP972095	N-deprived BG110 treated with silver nanoparticles (AgNPs), pH 7.5, 40 µmol photons m^{-2} s^{-1}	N/A	242 ml l^{-1}	[46]
Chlorella protothecoides	S-deprived TAP plus 0.35 mM NH4Cl, 25˚C, 40–50 µmol photons	5.53 ml l^{-1} h^{-1}	N/A	[7]

contrast, *Chlorella* sp. KLSc59 and *Chlorella* sp LSD-W2 gave the highest H_2 production under a light intensity of only 53.2 and 60 µmol photons m^{-2} s^{-1} [4,52]. Thus, light-dependent H_2 production varies based on the type of strains and species. Excessive light intensities can result in cell damage by contributing to heat accumulation within the cultivation system, potentially leading to cell death [10].

In this study, *Chlorella* sp. ChiW1 showed the highest H_2 production rate with 31.28 ± 1.73 µmol H_2 mg chl^{-1} h^{-1} or 15.32 ± 0.45 ml l^{-1} h^{-1} and reached the maximum H_2 production yield with 925.32 ± 19.95 µmol H_2 mg chl^{-1} or 448.98 ± 5.77 ml l^{-1}. These results were observed in cells grown in TAP medium for 36 hours, subsequently incubated in N-deprived TAP medium for 2 days before harvesting the cells and adjusting the cell density to an OD_{750} of about 2.0 in N-deprived TAP medium containing 175 mmol C-atom l^{-1} acetic acid at pH 7.2, and incubating at 35°C under light intensity of 150 µmol photons m^{-2} s^{-1} (Table 6). These H_2 production rates and yields were higher than those of other *Chlorella* strains, due to the strain dependence and environmental factors, including medium composition, medium pH, incubation temperature, and light intensity [2,4,5,7,42–46]. Therefore, using conditions that are appropriate and harmonious within the system is crucial in developing a more efficient and sustainable H_2 production process.

5. CONCLUSION

In summary, the green alga *Chlorella* sp. ChiW1, isolated from a rice paddy field in Chai Nat province, Thailand, demonstrated significant potential for efficient H_2 production compared to other investigated *Chlorella* strains. Nitrogen deprivation doubled H_2 production in *Chlorella* sp. ChiW1 by enhancing H_2ase activity due to reduced O_2 levels. Acetic acid, at a concentration of 175 m mol C-atom l^{-1} (or 87.5 mM), was identified as the optimal carbon source for H_2 production by *Chlorella* sp. ChiW1. Light intensity also played a crucial role in H_2 production. Future research directions for improving H_2 production by *Chlorella* sp. ChiW1 should focus on scaling up the process, which may be challenged by the reliance on nitrogen deprivation. Additionally, the supplementation of acetic acid and exposure to high light intensity should be optimized to enhance economic feasibility.

6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

11. PUBLISHER’S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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